REMARKS

This Amendment After Final is submitted in reply to the Office Action mailed on June 3, 2004. In the Office Action, the Examiner withdrew newly submitted claims 31-40 from consideration as allegedly being directed to a non-elected invention. Also in the Office Action, the Examiner rejected claims 1-5 and 13-30. In this Amendment After Final, claims 1-5 and 31-40 are canceled, claims 13-30 are amended, and new claims 41-83 are added. Upon entry of this Amendment After Final, the above-identified application will include claims 13-30 and 41-83.

Though claims 1-5 and 31-40 are canceled via this Amendment After Final, Applicants continue to believe that claims 1-5 and 31-40 are allowable, as originally presented in the above-identified application and as these claims presently exist as of the present request to cancel these claims. Likewise, though claims 13-30 are amended via this Amendment After Final, Applicants continue to believe that claims 13-30 are allowable, as originally presented in the above-identified application and as these claims presently exist as of the present request to amend these claims. Therefore, Applicants are canceling claims 1-5 and 31-40 and amending claims 13-30 without prejudice to Applicants' right to pursue claims worded like claims 1-5 and 13-40, as originally presented or as worded subsequent to original presentation, in the above-identified application or in a continuation application that is based on the above-identified application.

Furthermore, no claim amendment made herein is related to any statutory patentability requirement unless expressly stated herein. Also, no claim amendment made herein is made for the purpose of limiting (narrowing) the scope of any claim.

Examiner's Designation of the Present Office Action as Final

The Examiner's designation of the present Office Action as final based on the Amendment filed on February 23, 2004 is improper since the Examiner introduced a new ground of rejection in the present Office Action when rejecting claims 1-5 under the first paragraph of 35 U.S.C. §112. According to the Examiner:

> For clarification, as supported by the references provided by Applicant, an allelic variant of a given gene is a naturally occurring molecule which differs in sequence (by insertion, deletion, substitution). In other words, the point the Examiner was attempting to make was that an allelic variant is a product which occurs in nature and is not just any variant sequence of gene. There can sometimes be hundreds of allelic variants for a given gene and sometimes there are none.
>
> . . .
>
> At page 16 of the response, Applicant points to a definition of "allelic variant", and asserts that it is consistent with the terminology used in the disclosure. The Examiner does not disagree with the definition cited by Applicant, but again, must emphasize that "allelic variant" refers to a naturally-occurring molecule(s) with a precise nucleic acid sequence. This differs from any other variant which have modified for expression in bacteria. These nucleic acid molecules would be variant molecules, but in no way could they be construed as "allelic variant" within the art recognized meaning of the term, absent evidence to the contrary.

When the Examiner argued that an allelic variant is only a naturally-occurring molecule and only a product which occurs in nature, the Examiner introduced a new basis for rejecting claims 1-5 under the first paragraph of 35 U.S.C. §112. The fact that the Examiner has previously rejected claims 1-5 under the first paragraph of 35 U.S.C. §112 does not matter. The problem is the Examiner's characterization of an allelic variant was not presented in the prior Office Action, and therefore, Applicants were not given an opportunity to respond to the Examiner's improper characterization of allelic variants prior to the Examiner designating the Office Action as final. In essence, the Examiner's new argument regarding the allegedly "naturally-occurring" nature of allelic variants is akin to the Examiner relying upon a new reference in a prior art rejection. This new basis for rejecting claims 1-5 arose despite the fact that claims 1-5 were not amended in reply to the prior Office Action.

The Examiner introduced a new basis for rejecting claims 1-5 under the first paragraph of 35 U.S.C. §112 that was not necessitated by Applicants' amendment of claims 1-5 nor based on information newly submitted in an information disclosure statement. Consequently, the Examiner's

designation of the present Office Action as final is erroneous. Therefore, Applicants respectfully request that the Examiner reconsider and withdraw the finality of the present Office Action.

Examiner's Objection to the Claims

In the Office Action, the Examiner objected to claims 17 and 18 under 37 C.F.R. §1.75(c) as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. According the Examiner:

> The instant claims depend from base claims which have two requirements: 1) the DNA molecule must encode a porcine adipocyte leptin and 2) must hybridize to a specified sequence. The dependent claims 17-18 place size limitations on the DNA of 'at least 20' or 'at least 50 bases,' which is no where near the necessary size of a DNA which will encode a porcine leptin polypeptide, absent evidence to the contrary. Therefore, the claims do not appear to further limit the claims from which they depend.

Despite the Examiner's comments, Applicants respectfully disagree with the Examiner's characterization of claims 17 and 18 and with the Examiner's objection to claims 17 and 18.

Claims 17 and 18 each depend from independent claim 13. Independent claim 13 reads as follows:

> *13. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a porcine adipocyte polypeptide leptin that hybridizes to a nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

Therefore, claim 13 defines, in part, an isolated DNA molecule encoding porcine leptin that hybridizes to the nucleotide sequence identified as SEQ ID NO:1 under stringent hybridization conditions. Dependent claims 17 and 18 read as follows:

> *17. (Previously added) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is at least about 20 bases.*
>
> *18. (Previously added) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is at least about 50 bases.*

Hence, claim 17 specifies the isolated DNA molecule that encodes for porcine leptin polypeptide possess a length of at least about 20 bases. Similarly, claim 18 specifies the isolated DNA molecule that encodes for porcine leptin polypeptide possess a length of at least about 50 bases.

The Examiner's comments suggest it is improper for claims 17 and 18 to define structural features of the isolated DNA in terms of functional attributes by stating isolated DNA molecules which encode for porcine leptin polypeptide and are at least about 20 or at least about 50 bases in length. Nevertheless, the Examiner's characterization of the "at least about 20 bases" and the "at least about 50 bases" as a "size limitation" is inaccurate for several important reasons.

First, it is noted that definition of isolated DNA molecules having at least about 20 or at least about 50 bases (nucleotides) and which encode porcine leptin polypeptide, as defined in claims 17 and 18, is fully supported by the above-identified application. See, for example, [0042] of the published form of the above-identified application and column 5, lines 40-56 of U.S. Patent No. 6,277,592, issued to Bidwell at el. (referred to herein as "the Bidwell patent").

Next, the Examiner's characterization is inaccurate since claims 17 and 18 contain the term "at least," which is an open ended term requiring that the isolated DNA molecules have a minimum of about 20 bases and about 50 bases, respectively. Furthermore, a person of ordinary skill in the art would typically know to use the isolated DNA sequences having at least about 20 bases to perform hybridization experiments involving porcine leptin DNA sequences. (See the "Kennes publication of Exhibit A of this Amendment After Final). Additionally, those of ordinary skill in the art know that increasing the sequence length from at least about 20 bases to at least about 50 bases permits attainment of a higher specificity during hybridization experiments, as disclosed in the Bidwell patent and the above-identified application.

Finally, it is noted the term "at least" means "at a minimum" and therefore, the isolated DNA molecules claimed in claims 17 and 18 are correctly defined as having at least about 20 bases or at least about 50 bases in order to practice the invention of the above-identified application. The term "at least" is not intended to limit the isolated DNA sequences to only about 20 bases or to only about 50 bases in length, as the Examiner argues. Furthermore, the term "at

least" is not intended to suggest that porcine leptin polypeptide can be obtained from a DNA molecule containing only about 20 bases or only about 50 bases in length, as the Examiner alleges.

The Examiner's stated basis for rejecting claims 17-18 under 37 C.F.R. §1.75(c) is inaccurate and erroneous. Consequently, Applicants respectfully ask the Examiner to reconsider and withdraw the objection to claims 17-18 under 37 C.F.R. §1.75(c) and that claims 17-18 be allowed.

Claim Rejections Under the Written Description Requirement of the First Paragraph of 35 U.S.C. §112

In the Office Action, the Examiner rejected claims 1-5 and 13-30 under the first paragraph of 35 U.S.C. §112 for allegedly failing to provide an adequate written description. In support of this rejection, the Examiner stated:

> Claims 1-5 are rejected and newly added claims 13-30 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement for the reasons of record in the Office action mailed 22 September 2003. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Examiner's reasons of record "in the Office action mailed 22 September 2003" that are referenced in the recited paragraph from the present Office Action (which refers to claims 1-5, but not to claims 13-30 that were added after the September 22, 2003 mailing date of the prior Office Action) state, in part:

> Claims 1-5 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.
>
> In so far as the instant claims are directed to allelic variants of SEQ ID NO:1, the specification lacks an adequate written description of this subject matter. The recitation of 'allelic variant' is directed to a specific molecule for which the instant specification fails to describe the molecule in such a way as to reasonably convey to one skilled in the

> relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The structure of an 'allelic variant' cannot be predicated on the basis of the nucleotide sequence of SEQ ID NO:1 since there is no disclosure of where the variation occurs in the sequence of SEQ ID NO:1. The claims are directed to a species of nucleic acid, the structure of which cannot be determined or predicted from the disclosed nucleic acid sequence and the specification does not evidence isolation or conception of the structure of an 'allelic variant', therefore the specification does not provide an adequate written description of the claimed subject matter, and thus the claimed invention, to the extent that it reads upon an 'allelic variant' was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

As noted above, claims 1-5 have been canceled. Next, Applicants note the Examiner's only stated basis for rejecting claims 13-30 under the written description requirement of the first paragraph of 35 U.S.C. §112 was the "allelic variant" terminology. Claims 19-21 and 28 are believed allowable, since claims 19-21 and 28 have been amended such that claims 19-21 and 28 no longer recite the "allelic variant" terminology and therefore moot the Examiner's stated basis for rejecting claims 19-21 and 28 under the written description requirement of the first paragraph of 35 U.S.C. §112. However, claims 13-18, 22-27, and 29-30 did not previously and do not presently recite the "allelic variant" terminology of concern to the Examiner. Therefore, since there is not stated basis for rejecting claims 13-18, 22-27, and 29-30 under the written description requirement of the first paragraph of 35 U.S.C. §112, claims 13-18, 22-27, and 29-30 are likewise believed allowable.

Claims 13-30 are believed allowable. Therefore, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 13-30 under the written description requirement of the first paragraph of 35 U.S.C. §112 and that claims 13-30 be allowed.

Though claims 1-5 are canceled via this Amendment After Final, Applicants continue to believe claims 1-5 are allowable, as originally presented in the above-identified application and as these claims presently exist as of the present request to cancel these claims. Likewise, though claims 19-21 and 28 are amended via this Amendment After Final, Applicants continue to believe

that claims 19-21 and 28 are allowable, as originally presented in the above-identified application and as these claims presently exist as of the present request to amend these claims. Therefore, Applicants are canceling claims 1-5 and amending claims 19-21 and 28 without prejudice to Applicants' right to pursue claims worded like claims 1-5 and 19-21 and 28, as originally presented or as worded subsequent to original presentation, in the above-identified application or in a continuation application that is based on the above-identified application.

Claim Rejections Under the Enablement Requirement of the First Paragraph of 35 U.S.C. §112

In the Office Action, the Examiner rejected claims 14-15 and 17-20 under 35 U.S.C. §112, first paragraph, as allegedly failing to satisfy the enablement requirement. In support of this rejection, the Examiner stated:

> The claim(s) contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.
>
> The instant claims are directed to nucleic acid molecules which encode a porcine leptin polypeptide, wherein the nucleic acid hybridizes to at least 20-50 bases of SEQ ID NO:1, 20-50 bases of SEQ ID NO:3, or wherein the nucleic acid molecule is at least 20-50 bases long. First, the art does not recognize a nucleic acid as short as 20-50 nucleotides long that encodes a porcine leptin molecule and the instant specification fails to teach a molecule meeting this limitation. Therefore, one of ordinary skill in the art would not find such a length sufficient for encoding a leptin molecule from pigs, absent evidence to the contrary, and the claims are not enabled for such. Next, SEQ ID NO:1 is a genomic sequence with significantly long stretches of non-coding regions. Claims 14-15 indicate that the isolated DNA will hybridize to at least 20 or 50 nucleotides of SEQ ID NO:1, however, the vast majority of the nucleic acid molecules which hybridize (no conditions are provided, so the majority of nucleic acids in existence would hybridize under various conditions) to 20 or 50 bases would not meet the functional requirements of the claims, which are to encode a porcine leptin polypeptide. To suggest that one could then test each molecule for functional activity is not an enabling disclosure since the majority of

> nucleic acids from the pig would hybridize (DNA is inherently sticky) but would not be expected to encode a leptin molecule. Therefore, the claims are not enabled.

Despite the Examiner's comments, claims 14-15 and 17-20 are enabled by the disclosure in accordance with the first paragraph of 35 U.S.C. §112.

The Examiner's rejection of claims 14-15 and 17-20 under the first paragraph of 35 U.S.C. §112 is concerned with the enablement requirement of the first paragraph of 35 U.S.C. §112. The middle portion of the first paragraph of 35 U.S.C. §112 addresses the enablement requirement:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms, as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same . . . .

The enablement requirement is thus concerned with whether the specification disclosure teaches how to make and use the invention defined in the claims.

If the specification disclosure contains this teaching, the claims must be considered to be enabling under the first paragraph of §112 unless the Examiner explains why the Examiner doubts the truth or accuracy of any enabling statement in the disclosure. Otherwise stated, the Examiner has the initial burden of "setting forth a reasonable factual explanation, based on the record as a whole, as to *why* the Examiner believes the scope of protection provided by the claims is not adequately enabled by the description of the invention that is defined in the claims." In re Wright, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993). Furthermore, under Wright, the Examiner must back up assertions controverting the truth and accuracy of enabling statements with acceptable evidence or reasoning as to why the enabling statement is believed untrue or inaccurate.

Applicants assert the disclosure of the above-identified application does in fact enable claims 14-15 and 17-20. The above-identified application discloses porcine leptin polypeptide and nucleic acid molecules that encode porcine leptin polypeptide. The application further discloses (1) methods of isolating and identifying genes and oligonucleotides that encode for porcine leptin polypeptide and methods for using the isolated genes and oligonucleotides (DNA sequences) to

synthesize porcine leptin polypeptides. See column 6, lines 1-44, Example I, and Example III of the Bidwell patent, and [0011], [0014], [0015] and [0045] - [0049] of the published version of the above-identified application. The application also discloses methods of determining the susceptibility of a pig to fat deposition using, for example, the genes, oligonucleotides and polypeptides that encode for porcine leptin. See column 3, lines 7-20 and column 9, line 64, to column 10, line 6, of the Bidwell patent and [0017] and [0068] of the published version of the above-identified application.

Claims 14 and 15 each depend from independent claim 13. Independent claim 13 reads as follows:

> *13. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a porcine adipocyte polypeptide leptin that hybridizes to a nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

Therefore, claim 13 defines, in part, an isolated DNA molecule encoding porcine leptin that hybridizes to the nucleotide sequence identified as SEQ ID NO:1 under stringent hybridization conditions. Claims 14 and 15 each depend from independent claim 13 and read as follows:

> *14. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule hybridizes to at least 20 bases of the nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

> *15. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule hybridizes to at least 50 bases of the nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

Thus, dependent claims 14 and 15 specify that the isolated DNA molecules hybridize to at least about 20 bases and to at least about 50 bases of the nucleotide sequence identified as SEQ ID NO:1, respectively, under stringent hybridization conditions.

Next, claims 17 and 18 each depend from independent claim 13 and read as follows:

> *17. (Previously added) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is at least about 20 bases.*

18. (Previously added) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is at least about 50 bases.

Hence, claims 17 and 18 specify the isolated DNA molecules that encode for porcine leptin polypeptide possess a length of at least about 20 bases and a length of at least about 50 bases, respectively.

Next, claims 19 and 20 each dep end from independent claim 13 and read as follows:

19. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is capable of hybridizing to at least about 20 bases of a nucleotide sequence of SEQ ID NO:3 under stringent hybridization conditions.

20. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is capable of hybridizing to at least about 50 bases of a nucleotide sequence of SEQ ID NO:3 under stringent hybridization conditions.

Thus, claims 19 and 20 specify that the isolated DNA molecules hybridize to at least about 20 bases and to at least about 50 bases of the nucleotide sequence identified as SEQ ID NO:3, respectively, under stringent hybridization conditions.

In support of the rejection, the Examiner alleged, without any evidentiary support, "the art does not recognize a nucleic acid as short as 20-50 nucleotides long that encodes a leptin molecule." This statement of the Examiner, besides lacking evidentiary support, is erroneous. Indeed, the scientific literature recognizes a nucleic acid molecule as short as 17 nucleotides long that is based on the porcine leptin gene that encodes a porcine leptin molecule used to amplify portions of other porcine leptin genes. More specifically, as disclosed in the second paragraph of the article by Y.M. Kennes, B.D. Murphy, F. Pothier and M.-F Palin, entitled Characterization of Swine *Leptin* (*Lep*) Polymorphisms and Their Association with Production Traits (2001), primer sequences (nucleic acid molecules) 17 to 21 nucleotides long were used to amplify portions of the porcine leptin genes present in Landrace pigs. (Attached as Exhibit A of this Amendment After Final and referred to herein as the "Kennes publication").

The primer sequences of the Kennes publication were derived from the sequence identified as SEQ ID NO:1 in the Bidwell patent and in the above-identified application which encodes porcine leptin polypeptide; the primer sequences disclosed in the Kennes publication were 17, 21 and 20 nucleotides long, respectively. Thus, despite the Examiner's allegation to the contrary, the scientific literature does indeed recognize nucleic acid molecules having at least about 20 bases of a nucleotides sequence derived from a porcine leptin gene that encodes a porcine leptin molecule.

Next, contrary to the Examiner's comments ("the instant specification fails to teach a molecule meeting this limitation"), the specification of the above-identified application does indeed teach a nucleic acid molecule possessing at least about 20 nucleotides, as defined in claims 14, 17, and 19; for example, the specification discloses at [0011] of the published version of the present application and at column 2, lines 26-31 of the Bidwell patent:

> "the DNA molecule is preferably a single or double stranded DNA molecule having a nucleotide sequence consisting essentially of at least about 20 nucleotides of the nucleotide sequence depicted in Figures 1A-1D (SEQ ID NO:1)"

The Examiner's comments with regard to claims 14, 17, and 19 and with regard to claims 15, 18, and 20 are further contradicted by the specification disclosure starting at [0042] of the published version of the present application and at column 5, lines 50-56 of the Bidwell patent:

> "The DNA sequence should preferably have about 20 or more nucleotides to allow hybridization to another polynucleotide. In order to achieve higher specificity of hybridization, characterized by the absence of hybridization to sequences other than those encoding the polypeptide or a functional derivative thereof, a length of at least about 50 nucleotides is preferred.

Furthermore, the Bidwell patent (at column 6, lines 21-24) and the published version of the above-identified application (at [0047]) state:

> "Oligonucleotides representing a portion of the porcine adipocyte polypeptide are useful for screening for the presence of genes encoding such proteins and for the cloning of porcine adipocyte polypeptide genes.

Consequently, the specification of the present application clearly and indisputably teaches oligonucleotides (nucleic acid molecules) having a sequence length of at least about 20 bases and at least about 50 bases are useful for the identification and cloning of additional porcine DNA sequences and polypeptides that have homologous sequences to the sequences of the porcine leptin polypeptides disclosed in the present application.

The Examiner's enablement rejection of claims 14-15 and 17-20 is based on the faulty and unsupported premise that "the art does not recognize a nucleic acid as short as 20-50 nucleotides long that encodes a leptin molecule." This premise is wrong, as established above. Furthermore, the present application teaches, as established above, nucleic acid molecules with a sequence length of at least about 50 bases, and even as short as at least about 20 bases, are useful for identifying and cloning additional porcine DNA sequences and polypeptides with sequences homologous to the porcine leptin polypeptides disclosed in the present application.

Consequently, claims 14-15 and 17-20 are clearly enabled by the specification of the above-identified application. The specification of the present application and even the scientific literature disclose such oligonucleotides on the order of about 20 bases long, or more, and teach use of such molecules in hybridization studies.

Additionally, the Examiner's comments regarding testing isolated DNA molecule having at least about 20 nucleotides for functional porcine leptin activity are erroneous; therefore, these comments of the Examiner do not provide support for the Examiner's rejection of claims 14-15 and 17-20 under the enablement requirement. The Examiner's comments on this point are erroneous since the Examiner suggests the only application for using DNA sequences that encode for porcine leptin activity is generation of functional porcine leptin polypeptide. As noted above and as disclosed in the present application, DNA sequences of at least about 20 nucleotides are useful for isolating and identifying genes and oligonucleotides that encode for porcine leptine polypeptides. Also, as noted above and as disclosed in the present application, DNA sequences of at least about 20 nucleotides are useful for determining the susceptibility of a pig to fat deposition. The comments

demonstrate the Examiner's enablement rejection is faulty and without basis and that claims 14-15 and 17-20 are in fact enabled by the specification of the above-identified application.

Applicants have demonstrated that shown claims 14-15 and 17-20 are enabled by the specification of the above-identified application. The application teaches the generation and use of DNA sequences at least about 50 nucleotides long and even as short as at least about 20 nucleotides long. Applicants have provided factual evidence via the Kennes publication illustrating that DNA sequences with a length of at least about 20 nucleotides and based on the sequence identified as SEQ ID NO:1 are useful in hybridization trials involving porcine leptin gene. Furthermore, the present application enables use of DNA sequences of at least about 20 nucleotides for isolating and identifying genes and oligonucleotides that encode for porcine leptine polypeptides. Also, as noted above, the present application enables use of DNA sequences of at least about 20 nucleotides for determining the susceptibility of a pig to fat deposition.

Since the specification disclosure does contain an enabling disclosure of at least the same breadth as the scope of claims 14-15 and 17-20, the Examiner must consider claims 14-15 and 17-20 to be enabled under the first paragraph of §112 unless the Examiner explains why the Examiner doubts the truth or accuracy of any enabling statement provided in the above-identified application. The Examiner has the initial burden of "setting forth a reasonable factual explanation, based on the record as a whole, as to *why* the Examiner believes the scope of protection provided by the claims is not adequately enabled by the description of the invention that is defined in the claims." In re Wright. Furthermore, under Wright, the Examiner must back up assertions controverting the truth and accuracy of enabling statements with acceptable evidence or reasoning as to why the enabling statement is believed untrue or inaccurate. The Examiner has not demonstrated that the present invention, as defined in claims 14-15 and 17-20, is broader than the scope of the enabling disclosure. Furthermore, the Examiner has not produced any evidence that controverts the veracity of any specification statement that enables the present invention, as defined in claims 14-15 and 17-20. Therefore, claims 14-15 and 17-20 are believed enabled by the specification of the above-identified application.

Claims 14-15 and 17-20 are believed allowable. Therefore, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 14-15 and 17-20 under the enablement requirement of the first paragraph of 35 U.S.C. §112 and that claims 14-15 and 17-20 be allowed.

Claim Rejections Under the Second Paragraph, 35 U.S.C. §112

In the Office Action, the Examiner rejected claims 13-30 under the second paragraph of 35 U.S.C. §112 as allegedly "being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." The Examiner's specific remarks in regard to this rejection under the second paragraph of 35 U.S.C. §112 were:

> Claims 13-30 are directed to nucleic acid molecules (DNA, mRNA) which "hybridizes" to a particular disclosed nucleic acid sequence, wherein no hybridization conditions are provided. Some of the claims recite that a certain number of bases will hybridize (at least 20, at least 50), or that "substantially" all of the bases will hybridize, or "under hybridizing conditions". However, these claims are indefinite for the failure to indicate what hybridization conditions are to be used or what degree of identity is intended with "substantially all". Without knowing what conditions are to be used, the skilled artisan would not know if a molecule which may be isolated by using the disclosed nucleic acid molecule will be encompassed by the claims because the metes and bounds of what is claimed is not clear.

Despite the Examiner's allegations, claims 13-30 are believed definite in accordance with the second paragraph of 35 U.S.C. §112.

The Examiner's comments relate to the "under hybridizing conditions" terminology used in some of claims 13-30 and to the "substantially all" terminology used in some of claims 13-30. Claims employing the "substantially all" terminology are addressed first.

Claims 16, 21, 23-24, and 26-29 each employ the "substantially all" terminology of concern to the Examiner. The comments provided below in support of the definiteness of claim 16 that employs the "substantially all" terminology are equally applicable to claims 21, 23-24, and 26-29.

Claim 16 depends from independent claim 13 which reads as follows:

> *13. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a porcine adipocyte polypeptide leptin that hybridizes to a nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

Continuing, claim 16 reads as follows:

> *16. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule hybridizes to substantially all of the bases of the nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

Thus, claim 16 recites an isolated single or double-stranded DNA molecule which encodes a porcine adipocyte polypeptide leptin that hybridizes to substantially all of the bases of the nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.

The second paragraph of 35 U.S.C. §112 is concerned with whether those skilled in the art will be able to understand with a reasonable degree of accuracy what subject matter is circumscribed by the invention that is defined by a particular claim. If those skilled in the art can reasonably determine whether any particular subject matter either falls within the scope of a particular claim or falls outside the scope of the particular claim, that claim is not indefinite or ambiguous under the second paragraph of 35 U.S.C. §112. Miles Labs v Shandon, 27 USPQ2d 1123, 1123 (Fed Cir. 1993). Furthermore, the mere fact that the "substantially all" terminology of claim 16 is broader that stating a specific numeric determinant does not render a claim containing this language indefinite. See In re Miller, 441 F.2d 689, 169 USPQ 597 (CCPA 1971).

Applicants note that applications handled by the Examiner have issued into U.S. patents with claims than employ "substantially all" terminology. For example, claim 1 of U.S. Patent No. 6,756,484 employs the "substantially all" terminology more than five different times. In one instance, claim 1 recites:

> (C) . . . with a sufficient quantity of a first cation exchange elution buffer, which has a sufficiently high pH or ionic strength to displace **substantially all** of said authentic and non-authentic IGF-I from said cation exchange matrix . . . .

Emphasis added. Applicants' review of U.S. Patent No. 6,756,484 did not identify any particular numeric meaning or degree of identity for this use of the "substantially all" terminology. Indeed, the Courts do not require that any particular numeric meaning be provided for claim containing the "substantially all" terminology to be definite. The Examiner asks "what degree of identity is intended" for Applicants' use of the "substantially all" terminology in the claims at issue. However, like numeric meaning, the Courts do not require that any "degree of identity" be provided for a claim containing the "substantially all" terminology to be definite. Rather, the question is whether those skilled in the art will be able to understand with a *reasonable* degree of accuracy what subject matter is circumscribed by the invention that is defined by a particular claim, such as claim 16. Also, the issue is not whether the particular terminology is definite, but rather whether the meaning of the claim containing the terminology at issue is definite.

Some information about the meaning of the "substantially all" terminology is readily evident from the word itself. For example, "substantially all" clearly means something less than "all." Also, it is safe to say the "substantially all" means something more than "half." Beyond this, the "under stringent hybridization conditions" terminology of claim 16 provides further guidance.

Nucleic acid hybridization entails base pairing between two nucleic acid molecules, such as a single-stranded nucleic acid molecule of a defined sequence (probe) and a second nucleic acid molecule (target) with a sequence complementary to the sequence of the probe, to form a hybrid (duplex) molecule. Hybridization with Analysis of DNA Blots. Current Protocols in Molecular Biology, Section II 2.10.1. (John Wiley & Sons 2000) (attached as Exhibit B of this Amendment After Final); and Nucleic Acid Hybridization, pp 8-1 to 8-4, obtained at http://dir.niehs.nih.gov/dirlep/files/hybridiz.pdf on 11-19-04 (attached as Exhibit C of this Amendment After Final). The stability of the hybrid molecule formed by virtue of the hybridization depends on the extent of base-pairing that occurs between the target and the probe. (Exhibit B and Exhibit C of this Amendment After Final). Therefore, a high degree of base-pairing between the target and the probe results in a more stable hybrid wherein the target and the probe remain associated with each other; conversely, a low degree of base-pairing between target and probe

results in a less stable hybrid (duplex) wherein the target and the probe have an increased tendency to dissociate from each other. (Exhibit B and Exhibit C of this Amendment After Final).

Furthermore, those of ordinary skill in the art know a high degree of base-pairing in a hybrid molecule occurs when both target and probe possess sequences that are well-matched (complementary) to each other. (Exhibit B and Exhibit C of this Amendment After Final). In addition, a person of ordinary skill in the art knows little or no mismatch (pairing of non-complementary sequences) occurs between a probe and a target when stringent conditions are used during hybridization. (Exhibit B and Exhibit C of this Amendment After Final). This is because stringent hybridization conditions will allow only well-matched ("perfect or near perfect") hybrid molecules to form where a high degree of base-pairing is present between the DNA probe and the target DNA molecule. (Exhibit B of this Amendment After Final, also see Exhibit C of this Amendment After Final).

Claims 16, 21, 23-24, and 26-29 that each employ the "substantially all" terminology of concern to the Examiner also recite "stringent hybridization conditions." As noted above, one of ordinary skill in the art knows that under stringent hybridization conditions, a high degree of base-paring of the DNA probe to the target DNA molecule will result and only well-matched ("perfect or near perfect") hybrid molecules based on the highly or fully complementary sequences of the DNA probe and the target DNA molecule will form. Consequently, one of ordinary skill in the art would understand the "substantially all" term of claims 16, 21, 23, 24, and 26-29 characterizes the high ("perfect or near perfect") degree to which the DNA probe base-pairs to the target DNA molecule. The term "substantially all" is merely describes the high degree to which two DNA molecules are capable of hybridizing to each other under stringent hybridization conditions.

The foregoing comments demonstrate that one of ordinary skill in the art would be able to understand, with a *reasonable* degree of accuracy, what subject matter is circumscribed by the invention that is defined by claims 16, 21, 23-24, and 26-29 which employ the "substantially all" terminology. First, the meaning of the term "substantially all" clearly means something less than "all," yet more than "half." Beyond this, the "under stringent hybridization conditions" terminology

of claims 16, 21, 23-24, and 26-29 provide ample further guidance. Specifically, one of ordinary skill in the art would understand the "substantially all" term of claims 16, 21, 23, 24, and 26-29 characterizes the high ("perfect or near perfect") degree to which the DNA probe base-pairs to the target DNA molecule.

As noted, some of the Examiner's comments recited above relate to the "under hybridizing conditions" terminology used in some of claims 13-30. Claims 13-16 and 19-30 each recite hybridization or the capability of undergoing hybridization. Claims 17 and 18 depend from claim 13. The most pertinent comments of the Examiner in relation to the specified hybridization were:

> Claims 13-30 are directed to nucleic acid molecules (DNA, mRNA) which "hybridizes" to a particular disclosed nucleic acid sequence, wherein no hybridization conditions are provided. Some of the claims recite . . . "under hybridizing conditions." However, these claims are indefinite for the failure to indicate what hybridization conditions are to be used . . . .

Claims 13-16 and 19-30 now recite the hybridization occurs under stringent hybridization conditions. This is believed to adequately address the Examiner's rejection of claims 13-30 under the second paragraph of 35 U.S.C. §112 on the hybridization condition basis.

The above-identified application provides ample support for the 'stringent hybridization conditions" terminology recited in claims 13-16 and 19-30. A Declaration under 37 C.F.R. §1.132 by Dr. Michael Spurlock is attached. Exhibits A-P that are referenced in Dr. Spurlock's §1.132 Declaration are also attached as Exhibits A-P of the §1.132 Declaration. Dr. Spurlock is a co-inventor of the invention described and claimed in the above-identified application and is also a co-inventor of the invention defined and claimed in U.S. Serial Application No. 08/692,922, now U.S. Patent No. 6,277,592. ¶ 10 of attached §1.132 Declaration.

The invention of the above-identified application is, in one aspect, directed to nucleic acid molecules (and functional variants thereof) , such as (1) single or double-stranded DNA (and cDNA and genomic DNA) and (2) RNA (and mRNA), that encode porcine adipocyte polypeptide leptin. ¶ 19 of attached §1.132 Declaration. According to the present invention, the nucleic acid molecules (and functional variants thereof) encoding for porcine leptin polypeptide mentioned in

hybridize and are capable of hybridizing to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. ¶ 20 of attached §1.132 Declaration.

In another aspect, the invention of the above-identified application is directed to methods of hybridizing the nucleic acid molecules encoding for porcine leptin polypeptide (and functional variants thereof) to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. ¶ 21 of attached §1.132 Declaration. Examples II and III of the above-identified application provide particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention. ¶¶ 22 and 45 of attached §1.132 Declaration. This guidance of Examples II and III illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention. ¶¶ 22 and 45 of attached §1.132 Declaration.

The formation of a duplex by nucleic acid hybridization (base pairing between two nucleic acid molecules) is directly related to the degree of stringency of the hybridization conditions employed. ¶ 11 of attached §1.132 Declaration. Persons of ordinary skill in the art of molecular biology know high temperature is an example of a stringent hybridization condition. ¶ 12 of attached §1.132 Declaration. Persons of ordinary skill in the art of molecular biology know another example of a stringent hybridization condition is the chemical composition of the hybridization solution, such as hybridization solution with relatively low ionic strength (i.e. a relatively low salt concentration). ¶ 13 of attached §1.132 Declaration. Thus, persons of ordinary skill in the art of molecular biology know the stringency of hybridization conditions may be adjusted by varying the temperature at which hybridization is performed, the chemical composition of the hybridization solution used during hybridization experiments, or both the hybridization temperature and the chemical composition of hybridization solution. ¶ 14 of attached §1.132 Declaration.

Persons of ordinary skill in the art of molecular biology know time of exposure to hybridization solution at a particular temperature may also be manipulated to attain stringent hybridization conditions; for example, hybridization overnight for a time ranging from twelve to

sixteen hours is generally sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA or mRNA sequence. ¶ 15 of attached §1.132 Declaration. Persons of ordinary skill in the art of molecular biology also know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution. ¶ 16 of attached §1.132 Declaration. Persons of ordinary skill in the art of molecular biology know one example of non-specific DNA that may be used to block hybridization of non-specific DNA with the probe nucleic acid is salmon sperm. ¶ 17 of attached §1.132 Declaration.

Furthermore, beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments (including use of blocking substances as described above), those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

¶ 18 of attached §1.132 Declaration (citing Exhibit H of attached §1.132 Declaration. Thus, persons of ordinary skill in the art of molecular biology know the stringency of hybridization conditions may be adjusted by varying the temperature at which hybridization is performed, the chemical composition of the hybridization solution used during hybridization, the time of exposure to the hybridization solution at a particular temperature during hybridization, incorporation of non-specific DNA (such as salmon sperm) in the hybridization solution, and post-hybridization washing conditions, such as the temperature and/or salt concentration of the post-hybridization wash solution.

We first consider the hybridization conditions taught and disclosed by Example II of the above-identified application. Example II discloses a hybridization technique using porcine leptin cDNA as a probe to detect full length porcine leptin mRNA. ¶ 24 of attached §1.132 Declaration. According to Example II, porcine leptin cDNA was hybridized against porcine leptin mRNA at 60°C for fifteen

hours. ¶ 25 of attached §1.132 Declaration. WO 02/036829A2 states that a hybridization temperature of about 60°C is generally sufficient to establish stringent conditions for even long probes:

> Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7. 0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g., greater than 50 nucleotides).

¶ 26 of attached §1.132 Declaration (citing page 12, lines 2-6, of Exhibit I of §1.132 Declaration).

Furthermore, use of temperatures lower than 60°C (specifically, 50°C in numbered para. 4 on the first page of Exhibit K of §1.132 Declaration, 55°C in numbered para. 4 on the second page of Exhibit K of §1.132 Declaration, and 50°C in numbered para. 4 on the third page of Exhibit K of §1.132 Declaration) is disclosed as adequate to produce stringent conditions using a non-formamide hybridization solution. ¶ 27 of attached §1.132 Declaration (citing Exhibit K of §1.132 Declaration). The evidence provided above regarding use of a hybridization temperature of 60°C during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II. ¶ 28 of attached §1.132 Declaration. This statement is probative as to stringency even if the salt concentration in the hybridization solution of Example II is not taken into account. See ¶ 14 of attached §1.132 Declaration.

Next, we consider the hybridization time of fifteen hours employed in Example II. ¶¶ 23 and 25 of attached §1.132 Declaration. Hybridization overnight for a time ranging from twelve to sixteen hours is generally sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA or mRNA sequence. ¶ 30 of attached §1.132 Declaration (citing page 3 of Exhibit D of §1.132 Declaration). Others in the molecular biology industry also indicate hybridization overnight (specifically, numbered para. 4 on the first page of Exhibit K, numbered para. 4 on the second page of Exhibit K, and numbered para. 4 on the third page of Exhibit K) is adequate to produce stringent conditions using a non-formamide hybridization solution. ¶ 31 of attached §1.132 Declaration (citing Exhibit K of §1.132 Declaration). The evidence provided above regarding hybridization for fifteen

hours, which clearly qualifies as overnight, during the hybridization trial of Example II of the present application, illustrates to those of ordinary skill in the art of molecular biology, stringent hybridization conditions were employed during the hybridization trial of Example II. See ¶ 32 of attached §1.132 Declaration.

Next, we consider the salt concentration employed in the hybridization solution of Example II.. Since the hybridization solution contained 0.9 M NaCl and 0.09 M sodium citrate, one of ordinary skill in the art of molecular biology would understand the hybridization solution contained 0.99 M sodium ion. ¶ 33 of attached §1.132 Declaration (citing ¶ 23 of §1.132 Declaration). WO 02/036829A2 states that salt concentrations of 0.99 M sodium ion are considered to be low salt concentration generally sufficient to establish stringent conditions for even long probes:

> Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7. 0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g., greater than 50 nucleotides).

¶ 34 of attached §1.132 Declaration (citing page 12, lines 2-6, of Exhibit I of §1.132 Declaration). The evidence provided above regarding use of a hybridization solution with a salt concentration of 0.99 M sodium ion during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, that stringent hybridization conditions were employed during the hybridization trial of Example II. ¶ 35 of attached §1.132 Declaration. This statement is probative as to stringency even if the hybridization temperature of Example II is not taken into account. ¶ 35 of attached §1.132 Declaration (citing ¶ 14 of §1.132 Declaration).

Next, we consider the salmon sperm concentration of 100 mg/ml employed in the hybridization solution of Example II. ¶ 36 of attached §1.132 Declaration (citing ¶ 23 of §1.132 Declaration). Persons of ordinary skill in the art of molecular biology also know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution. ¶ 37 of attached §1.132 Declaration (citing ¶ 16 and Exhibit E of §1.132 Declaration). The non-specific DNA is said to prevent the probe from hybridizing nonspecifically - that is with nucleic acid

that is not complementary (specific) to the probe nucleic acid. ¶ 37 of attached §1.132 Declaration (citing ¶ 16 and Exhibit F of §1.132 Declaration).

As an example, Salmon sperm at a concentration of 100 mg/ml is incorporated by those skilled in the art of molecular biology to complement and support high stringency hybridization by limiting non-specific nucleic acid binding during hybridization. ¶ 38 of attached §1.132 Declaration (citing Exhibit J of §1.132 Declaration). The evidence provided above regarding use of a hybridization solution with a salmon sperm concentration of 100 mg/ml during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of conditions that inhibit non-specific probe hybridization and therefore further support attainment of results consistent with use of stringent conditions during the hybridization trial of Example II. ¶ 39 of attached §1.132 Declaration.

We next consider the post-hybridization washing conditions of Example II that entailed washing "to a final stringency of 0.2xSSC (0.03 M NaCl, 0.003 M sodium citrate), 0.1% SDS at 60°C. ¶ 40 of attached §1.132 Declaration (citing ¶ 23 of §1.132 Declaration). Beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments , those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions, such as the temperature and/or the salt concentration of the wash solution. ¶ 41 of attached §1.132 Declaration (citing ¶ 18 of §1.132 Declaration). For example:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

¶ 41 of attached §1.132 Declaration (citing Exhibit H of §1.132 Declaration).

In this regard, those of ordinary skill in the art recognize that high stringency washing may be accomplished using the hybridization temperature in combination with a final washing solution containing 0.2X SSC and 0.1% SDS, as was employed in Example II:

> Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2-0.1 times SSC, 0.1% SDS.

¶ 42 of attached §1.132 Declaration (citing ¶ 0188 of Exhibit L of §1.132 Declaration). The foregoing evidence demonstrates those of ordinary skill in the art of molecular biology recognize that high stringency washing may be accomplished using a final washing solution containing 0.2X SSC and 0.1% SDS, as was employed in Example II, and also demonstrates this stringent washing solution may be employed at the hybridization temperature to further enhance the washing stringency. ¶ 43 of attached §1.132 Declaration. Thus, the use of high stringency washing, as was employed in Example II of the above-identified application, illustrates, to those of ordinary skill in the art of molecular biology, use of conditions that further support attainment of results consistent with use of stringent conditions during the hybridization trial of Example II.

Example II of the above-identified application provides particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention. ¶ 22 of attached §1.132 Declaration. This guidance of Example II illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention. ¶ 22 of attached §1.132 Declaration. Furthermore, this guidance of Example II teaches and discloses use of stringent hybridization conditions for practicing the hybridization methods of the present invention.

For instance, Example II discloses use of a hybridization temperature of 60°C that those of ordinary skill in the art of molecular biology would recognize as a stringent hybridization condition. Also, Example II employs a hybridization solution with a salt concentration of 0.99 M sodium ion that those of ordinary skill in the art of molecular biology would recognize as a stringent hybridization condition. Also, those of ordinary skill in the art of molecular biology would recognize the Example II hybridization for fifteen hours as being consistent with stringent hybridization conditions. Continuing, those of ordinary skill in the art of molecular biology would recognize the use in Example II of a hybridization solution with a salmon sperm concentration of 100 mg/ml as being consistent with use of stringent hybridization conditions. Finally, those of ordinary skill in the art of molecular biology would recognize the use in Example II of stringent washing conditions (final washing solution at hybridization

temperature and containing 0.2X SSC and 0.1% SDS), as use of conditions that may be part of an overall stringent hybridization protocol that support attainment of results consistent with use of stringent conditions during the Example II hybridization trial.

The guidance of Example II regarding hybridization conditions illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention. Indeed, the evidence provided herein illustrates one of ordinary skill in the art of molecular biology would recognize that the hybridization conditions employed in Example II of the above-identified application constitute stringent hybridization conditions that would typically enable a probe, such as the porcine leptin cDNA molecule employed in Example II, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe. ¶ 44 of attached §1.132 Declaration (citing ¶¶ 22-43 of the §1.132 Declaration, as supplemented by Exhibits B-D of the §1.132 Declaration and by the Exhibits referenced in ¶¶ 22-43 of the §1.132 Declaration).

Next, we consider the hybridization conditions taught and disclosed by Example III of the above-identified application. Example II discloses a hybridization trial that used porcine leptin cDNA as a probe to screen a porcine genomic DNA library and detect porcine leptin DNA. ¶ 47 of attached §1.132 Declaration. According to Example III, the porcine leptin cDNA was hybridized against the porcine leptin DNA at 65°C overnight. ¶ 48 of attached §1.132 Declaration. WO 02/036829A2 states that a hybridization temperature of about 60°C is generally sufficient to establish stringent conditions for even long probes:

> Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7. 0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g., greater than 50 nucleotides).

¶ 49 of attached §1.132 Declaration (citing page 12, lines 2-6, of Exhibit I of §1.132 Declaration).

Furthermore, use of temperatures lower than 60°C (specifically, 50°C in numbered para. 4 on the first page of Exhibit K of §1.132 Declaration, 55°C in numbered para. 4 on the second page of Exhibit K of §1.132 Declaration, and 50°C in numbered para. 4 on the third page of Exhibit K of §1.132

Declaration) is disclosed as adequate to produce stringent conditions using a non-formamide hybridization solution. ¶ 50 of attached §1.132 Declaration (citing Exhibit K of §1.132 Declaration). The evidence provided above regarding use of a hybridization temperature of 65 °C during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example III. ¶ 51 of attached §1.132 Declaration (citing Exhibit K of §1.132 Declaration). This statement is probative as to stringency even if the salt concentration in the hybridization solution of Example III is not taken into account. (See ¶ 14 of attached §1.132 Declaration).

Next, we consider the overnight hybridization period employed in Example III. ¶¶ 46 and 52 of attached §1.132 Declaration. Hybridization overnight is generally recognized by those of ordinary skill in the art of molecular biology as being sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA sequence. ¶ 53 of attached §1.132 Declaration (citing page 3 of Exhibit D of §1.132 Declaration). Others in the molecular biology industry also indicate hybridization overnight (specifically, numbered para. 4 on the first page of Exhibit K, numbered para. 4 on the second page of Exhibit K, and numbered para. 4 on the third page of Exhibit K) is adequate to produce stringent conditions using a non-formamide hybridization solution. ¶ 54 of attached §1.132 Declaration (citing Exhibit K of §1.132 Declaration). The evidence provided above regarding hybridization overnight during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, stringent hybridization conditions were employed during the hybridization trial of Example III. (See ¶ 55 of attached §1.132 Declaration).

We next consider the post-hybridization washing conditions of Example III that concluded with a final wash containing "0.2x SSC, 0.5% SDS . . . at 60 °C for 30 min." ¶ 56 of attached §1.132 Declaration (citing ¶ 46 of §1.132 Declaration). Beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments , those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing

conditions, such as the temperature and/or the salt concentration of the wash solution. ¶ 57 of attached §1.132 Declaration (citing ¶ 18 of §1.132 Declaration). For example:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

¶ 57 of attached §1.132 Declaration (citing Exhibit H of §1.132 Declaration).

In this regard, those of ordinary skill in the art recognize that high stringency washing may be accomplished using the hybridization temperature in combination with a final washing solution containing 0.2X SSC and 0.5% SDS, as was employed in Example III:

> Filters were washed under stringent conditions (0.2x SSC, 0.5% SDS at 60°C).

¶ 58 of attached §1.132 Declaration (citing Exhibit M of §1.132 Declaration).

The foregoing evidence demonstrates those of ordinary skill in the art of molecular biology recognize that high stringency washing may be accomplished using a final washing solution containing 0.2X SSC and 0.5% SDS, as was employed in Example III. ¶ 59 of attached §1.132 Declaration. Thus, use of high stringency washing, as was employed in Example III of the above-identified application, illustrates, to those of ordinary skill in the art of molecular biology, use of conditions that further support attainment of results consistent with use of stringent conditions during the hybridization trial of Example III.

Example III of the above-identified application provides particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention. ¶ 45 of attached §1.132 Declaration. This guidance of Example III illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention. ¶ 45 of attached §1.132 Declaration. Furthermore, this guidance of Example III teaches and discloses use of stringent hybridization conditions for practicing the hybridization methods of the present invention.

For instance, Example II discloses use of a hybridization temperature of 65°C that those of ordinary skill in the art of molecular biology would recognize as a stringent hybridization condition. Also, those of ordinary skill in the art of molecular biology would recognize the Example III overnight

hybridization period as being consistent with stringent hybridization conditions. Additionally, those of ordinary skill in the art of molecular biology would recognize the use in Example III of stringent washing conditions (final washing solution containing 0.2X SSC and 0.5% SDS), as use of conditions that may be part of an overall stringent hybridization protocol that support attainment of results consistent with use of stringent conditions during the Example III hybridization trial.

The guidance of Example III regarding hybridization conditions illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention. Indeed, the evidence provided herein illustrates one of ordinary skill in the art of molecular biology would recognize that the hybridization conditions employed in Example III of the above-identified application constitute stringent hybridization conditions that would typically enable a probe, such as the porcine leptin cDNA molecule employed in Example III, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe. ¶ 60 of attached §1.132 Declaration (citing ¶¶ 45-59 of the §1.132 Declaration, as supplemented by Exhibits B-D of the §1.132 Declaration and by the Exhibits referenced in ¶¶ 45-59 of the §1.132 Declaration).

As noted above, in one aspect, the invention of the above-identified application is directed to a variety of nucleic acid molecules (and functional variants thereof) that encode porcine adipocyte polypeptide leptin and are capable of hybridizing to nucleotide sequences (and portions thereof). ¶ 61 of attached §1.132 Declaration (citing ¶¶ 19-21 of the §1.132 Declaration). In another aspect, the invention of the above-identified application is directed to hybridizing the nucleic acid molecules (and functional variants thereof) to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. ¶ 61 of attached §1.132 Declaration (citing ¶¶ 19-21 of the §1.132 Declaration). The present application, primarily via Examples II and III, discloses conditions for hybridizing the nucleic acid molecules addressed in the present application. ¶ 62 of attached §1.132 Declaration.

As explained above, one of ordinary skill in the art of molecular biology, upon reviewing the hybridization conditions employed in Examples II and III of the above-identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically

enable a probe, such as the porcine leptin cDNA molecule employed in Examples II and III, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe, such as porcine leptin mRNA or porcine leptin DNA. ¶ 62 of attached §1.132 Declaration. Consequently, since the above-identified application primarily discloses to one of ordinary skill in the art of molecular biology use of stringent hybridization conditions for hybridizing the nucleic acid molecules addressed in the present application, it is evident use of stringent hybridization conditions for hybridizing the nucleic acid molecules addressed in the present application is in fact disclosed and described in the present application. ¶ 63 of attached §1.132 Declaration.

Claims 13-30 are believed allowable. Therefore, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 13-30 under the second paragraph of 35 U.S.C. §112 and that claims 13-30 be allowed.

Claim Rejections Under 35 U.S.C. §103(a)

In the Office Action, the Examiner rejected claims 22 and 24-27 under 35 U.S.C. 103(a) as allegedly being unpatentable over U.S. Patent N. 6,309,853 to Friedman et al. (subsequently referred to as the "Friedman patent"). In support of this rejection, the Examiner stated:

> The instant claims are directed to isolated nucleic acids which encode porcine leptin and hybridize SEQ ID NO:3 or a 'functional derivative thereof' (see claims 22, 27) or 'variant'(see claims 24-26). The prior art of Friedman et al. (U.S. Pat. No. 6,309,853) disclose nucleic acids which encode human and mouse leptin, which would be considered functional derivatives and/or variants of SEQ ID NO:3 since they encode leptin molecules and would possess similar functional properties as those of the porcine leptin, absent evidence to the contrary. Friedman et al. teach that the leptin gene (or OB) could be isolated from domestic animals using the methods disclosed therein (see column 26, line 53 to column 27, line 49). Friedman et al. specifically mention swine as a domestic animal for which leptin would be useful (see column 48, lines 41-47). Friedman et al. do not specifically disclose an isolated nucleic acid encoding a porcine leptin polypeptide. However, it would have been obvious to use the nucleic acid of Friedman et al. encoding human or mouse leptin and hybridize it to a porcine DNA library and isolate a nucleic acid molecule encoding porcine leptin because Friedman et al. teach methods for

> isolating leptin encoding nucleic acids and also teach that it would be beneficial to administer leptin to swine. Therefore, the invention as a whole would have been obvious at the time it was made, absent evidence to the contrary.
>
> Applicant should note that the instant rejection is being made because the claims do not require the specifics of SEQ ID NO:1 or 3, and therefore, methods of isolating nucleic acids for leptin using a functional equivalent of porcine leptin encoding DNA encompasses methods using human or murine DNA encoding leptin.

Despite the Examiner's allegations, the Friedman patent does not teach, suggest, disclose, or make obvious the present invention as defined in claims 22 and 24-27.

Independent claim 22 reads as follows:

> *22. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a porcine adipocyte polypeptide leptin, the isolated DNA molecule consisting of a nucleotide sequence SEQ ID NO:3 or a functional derivative thereof, wherein the isolated DNA molecule or the functional derivative thereof hybridizes to the nucleotide sequence of SEQ ID NO:3 when placed in contact with the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.*

Claim 22 thus defines an isolated porcine leptin DNA molecule that contains SEQ ID NO:3 or a functional derivative of SEQ ID NO:3 and further specifies that the isolated porcine leptin DNA molecule or the functional derivative thereof hybridizes to SEQ ID NO:3 under stringent hybridization conditions.

Next, independent claim 24 reads as follows:

> *24. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a porcine adipocyte polypeptide leptin, the isolated DNA molecule consisting of a nucleotide sequence SEQ ID NO:3 or a variant thereof, wherein the isolated DNA molecule or the variant thereof hybridizes to substantially all of the nucleotide sequence of SEQ ID NO:3 when placed in contact with the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.*

Claim 24 thus defines an isolated porcine leptin DNA molecule that contains SEQ ID NO:3 or a variant of SEQ ID NO:3 and further specifies that the isolated porcine leptin DNA molecule or the variant thereof hybridizes to substantially all of SEQ ID NO:3 under stringent hybridization conditions.

Next, independent claim 25 reads as follows:

> *25. (Currently Amended) An isolated mRNA molecule which encodes a porcine adipocyte polypeptide leptin, the mRNA molecule encoded by a nucleotide sequence SEQ ID NO:3 or a variant of the mRNA molecule, wherein the mRNA molecule or the variant of the mRNA molecule hybridizes to the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.*

Claim 25 thus defines a porcine leptin mRNA molecule of SEQ ID NO:3 or a variant of SEQ ID NO:3 and further specifies that the isolated porcine leptin mRNA molecule or the variant thereof hybridizes to SEQ ID NO:3 under stringent hybridization conditions.

Finally, claim 27 reads as follows:

> *27. (Currently Amended) An isolated mRNA molecule which encodes a porcine adipocyte polypeptide leptin, the mRNA molecule encoded by a nucleotide sequence SEQ ID NO:3 or a functional derivative thereof, wherein the functional derivative of the isolated mRNA molecule hybridizes to substantially all of the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.*

Hence, claim 27, defines a porcine leptin mRNA molecule of SEQ ID NO:3 or a functional derivative of SEQ ID NO:3 or a functional derivative of SEQ ID NO:3 and further specifies that the isolated porcine leptin mRNA molecule or the functional derivative thereof hybridizes to substantially all of SEQ ID NO:3 under stringent hybridization conditions.

As noted above, the Friedman patent does not teach, suggest, or disclose the invention of the above-identified application, as defined in claims 22 and 24-27. Consistent with the Examiner's observation, the Friedman patent does disclose murine and human leptin DNA sequences and polypeptides. ¶ 67 of attached §1.132 Declaration. Also, consistent with the Examiner's observation,

the Friedman patent does not disclose any porcine leptin DNA (or mRNA) molecules or polypeptides. ¶ 68 of attached §1.132 Declaration. Furthermore, consistent withe the Examiner's observation, the Friedman patent does not disclose any functional derivative or variant DNA (or mRNA) molecules that encode for porcine leptin polypeptide. ¶ 69 of attached §1.132 Declaration.

Furthermore, the human and murine leptin DNA sequences disclosed in the Friedman patent differ substantially from the porcine leptin DNA sequences disclosed in the above-identified application; consequently, the porcine leptin of the present application that is based on the porcine leptin DNA sequences (and functional variants thereof) disclosed in the above-identified application differs in substantial detail from both the human leptin and the murine leptin disclosed in the Friedman patent. ¶ 70 of attached §1.132 Declaration. As a result, the porcine leptin of the present application is functionally different from both the human leptin and the murine leptin disclosed in the Friedman patent. ¶ 71 of attached §1.132 Declaration.

For example, when recombinant porcine leptin protein is administered by intracerebroventricular (ICV) injection to crossbred prepuberal gilts (normal female pigs), the crossbred prepuberal gilts exhibit increased growth hormone secretion after the leptin administration. ¶ 72 of attached §1.132 Declaration (citing Exhibit N of the §1.132 Declaration). See also ¶¶ 75-85 of attached §1.132 Declaration. On the other hand, when recombinant human leptin protein is administered by ICV injection to normal male rats, the normal male rats do not exhibit increased growth hormone secretion after the leptin administration. ¶ 73 of attached §1.132 Declaration (citing Exhibit O of the §1.132 Declaration). See also ¶¶ 86-95 of attached §1.132 Declaration.

Since recombinant porcine leptin protein administration increases growth hormone secretion in pigs, while recombinant human leptin protein administration fails to increase growth hormone secretion in male rats, the effects of porcine leptin protein administration and human leptin protein administration differ dramatically, and it is consequently evident the porcine leptin protein is functionally very different from the human leptin protein. ¶ 74 of attached §1.132 Declaration. Otherwise stated, based on the documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication), it is evident that

recombinant porcine leptin protein surprisingly functions very differently from recombinant human leptin protein upon administration to mammals. ¶ 97 of attached §1.132 Declaration (citing ¶¶ 73-74 and 96 of the §1.132 Declaration).

As another example, when recombinant porcine leptin is administered by intracerebroventricular (ICV) injection to crossbred prepuberal gilts (normal female pigs), the recombinant porcine leptin administration fails to change thyroxine (T_4) secretion. ¶ 98 of attached §1.132 Declaration (citing EXP I on pages 80-81 of Exhibit N of the §1.132 Declaration). See also ¶¶ 75-83 of attached §1.132 Declaration. On the other hand, when recombinant mouse leptin is administered by ICV injection to normal ad libitum fed male rats, the normal ad libitum fed male rats exhibit significantly decreased thyroxine (T_4) levels in the blood after the recombinant mouse leptin administration. ¶ 99 of attached §1.132 Declaration (citing Exhibit P of the §1.132 Declaration). See also ¶¶ 101-105 of attached §1.132 Declaration.

Since recombinant porcine leptin administration fails to change thyroxine (T_4) secretion in pigs, while recombinant murine leptin administration significantly decreased thyroxine (T_4) levels in the blood of male rats, the effects of porcine leptin administration and murine leptin administration differ dramatically, and it is evident the porcine leptin functions very differently from the murine leptin. ¶ 100 of attached §1.132 Declaration. Otherwise stated, based on the documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus thyroxine (T_4) secretion of normal fed rats (per the Cusin publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant murine leptin protein upon administration to mammals. ¶ 107 of attached §1.132 Declaration (citing ¶¶ 100 and 106 of the §1.132 Declaration).

The foregoing factual evidence illustrates the human leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary (see Paragraph 64 of attached §1.132 Declaration), necessarily, or actually, possess functional properties that are similar to the functional properties of the porcine leptin disclosed in the above-identified application. ¶ 108 of attached §1.132 Declaration. For example, based on the documented differences in growth hormone secretion after ICV

administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant human leptin protein upon administration to mammals. ¶ 109 of attached §1.132 Declaration (citing ¶¶ 72-74 and 96 of the §1.132 Declaration).

The documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Carro publication, that would suggest the differential effects on growth hormone secretion caused by porcine leptin protein administration in pigs versus human leptin protein administration in rats. ¶ 110 of attached §1.132 Declaration. Furthermore, the documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Carro publication, that would suggest human leptin protein administration in rats would have no effect on growth hormone secretion by the rats, while porcine leptin protein administration in pigs would cause an increase in growth hormone secretion by the pigs. ¶ 111 of attached §1.132 Declaration.

Therefore, based on the factual results noted above and despite the Examiner's contentions to the contrary (see ¶ 64 of attached §1.132 Declaration), the Examiner's speculative suggested hybridization of the nucleic acid of the Friedman patent that encodes human leptin to a porcine DNA library and subsequent isolation of a nucleic acid molecule encoding porcine leptin is not suggested. ¶ 112 of attached §1.132 Declaration (citing ¶¶ 106-111 of attached §1.132 Declaration). Any alleged suggestion fails to exist since the functional characteristics of human leptin disclosed in the Friedman patent would not confirm isolation of a nucleic acid molecule encoding for porcine leptin, as claimed in the above-identified application. ¶ 112 of attached §1.132 Declaration (citing ¶¶ 106-111 of attached §1.132 Declaration).

Likewise, the foregoing factual evidence illustrates the murine leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary (see of attached §1.132

Declaration), necessarily, or actually, possess functional properties that are similar to the functional properties of the porcine leptin disclosed in the above-identified application. ¶ 113 of attached §1.132 Declaration. For example, based on the documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Cusin publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant murine leptin protein upon administration to mammals. ¶ 114 of attached §1.132 Declaration (citing ¶¶ 98-100 and 106-107 of attached §1.132 Declaration).

The documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Cusin publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Cusin publication, that would suggest the differential effects on thyroxine (T_4) secretion caused by porcine leptin protein administration in pigs versus murine leptin protein administration in rats. ¶ 115 of attached §1.132 Declaration. Furthermore, the documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Cusin publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Cusin publication, that would suggest murine leptin protein administration in rats would reduce thyroxine (T_4) secretion in the rats, while porcine leptin protein administration in pigs would not affect thyroxine (T_4) secretion by the pigs. ¶ 116 of attached §1.132 Declaration.

Finally, despite the Examiner's contentions to the contrary (see Paragraph 64), the Examiner's speculative suggested hybridization of the nucleic acid of the Friedman patent that encodes mouse leptin to a porcine DNA library and subsequent isolation of a nucleic acid molecule encoding porcine leptin is not suggested. ¶ 117 of attached §1.132 Declaration. Any alleged suggestion fails to exist since the functional characteristics of murine leptin disclosed in the Friedman patent would not confirm isolation of a nucleic acid molecule encoding for porcine leptin as claimed in the above-identified application. ¶ 117 of attached §1.132 Declaration.

Substantial differences exist between the porcine leptin DNA (or mRNA) molecules of the above-identified application and the human and murine leptin DNA (or mRNA) molecules disclosed

in the Friedman patent. These substantial differences between the molecules are apparently (or may be) responsible for the surprising and unexpected differences in the functional activity of porcine leptin polypeptide when compared to human leptin and to murine leptin. No matter the cause of the surprising and unexpected differences in the functional activity, these surprising and unexpected differences illustrate the non-obvious nature of the present invention, as defined in claims 22 and 24-27, considering the Friedman patent. Furthermore, any suggestion to use the nucleic acid of the Friedman patent that encodes human or mouse leptin, hybridize it to a porcine DNA library, and isolate a nucleic acid molecule encoding porcine leptin per the Examiner's comments fails to exist. This failure is evident since the functional characteristics of human leptin and of murine leptin would not confirm isolation of nucleic acid molecules encoding for porcine leptin, as defined in claims 22 and 24-27 of the above-identified application.

Claims 22 and 24-27 are believed allowable. Consequently, Applicants respectfully request that the Examiner reconsider and withdraw the rejections of claims 22 and 24-27 under 35 U.S.C.§103(a) based on the Friedman patent and that pending claims 22 and 24-27 be allowed.

New Claims Added by Applicants

Applicants have added new claims 41-83. New claims 41-83 do not add any new matter to the above-identified application. New claim 41 depends from independent claim 13. Support for new claim 41 is believed to exist throughout the above-identified application, such as in Examples I and III. Support for new claims 42-45 is believed to exist throughout the above-identified application, such as in Examples I, II and III. New claims 46-49 depend from independent claims 22, 24, 25 and 27, respectively. Support for new claims 46-49 is believed to exist throughout the Bidwell patent, such as at column 5, lines 12-25; column 6, lines 28-44; column 6, lines 54-59; and in both the Bidwell patent and the above-identified application at Examples I, II and III. Applicants respectfully request consideration and allowance of new claims 41-83.

Specification Amendments Made By Applicants

Applicants have amended the above-identified application, as indicated above, to correct typographical errors and to separate nucleic acid sequence listings from amino acid sequence listings Amendments to the Sequence Listing. The Amendments, as they relate to the Sequence Listing, are accompanied by two copies of a computer readable form of the Sequence Listing in compliance with § 1.52(e). Finally, no amendment to the Specification, Sequence Listings or the Computer Readable Form of the Sequence Listings includes new matter.

CONCLUSION

Claims 13-30 and 41-83 are believed allowable. Therefore, reconsideration and allowance of claims 13-30 is respectfully requested. Likewise, consideration and allowance of new claims 41-83 is respectfully requested. The Examiner is invited to contact Applicants' below-named attorney, Philip F. Fox, to facilitate allowance of the above-identified application.

Respectfully submitted,
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Date: November 23, 2004

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Appln. No.	:	09/932,888	
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Title	:	Porcine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0016	

EXHIBIT A

OF

AMENDMENT AFTER FINAL

Y.M. Kennes, B.D. Murphy, F. Pothier and M.-F Palin, entitled

Characterization of Swine *Leptin* (*Lep*) Polymorphisms

and Their Association with Production Traits (2001)

SHORT COMMUNICATION

Characterization of swine *leptin* (*LEP*) polymorphisms and their association with production traits

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Summary

Four polymorphisms in the swine *leptin* (*LEP*) gene were characterized and evaluated for association with economically important production traits in Yorkshire, Landrace and Duroc pigs. Our results show that these polymorphisms are generally of low frequency or are absent in pig populations. Two polymorphisms (A2845T and T3469C) may be associated ($P < 0.0078$) with feed intake and growth rate traits in Landrace pigs.

Keywords *LEP* gene, leptin, pig, polymorphism.

Leptin, the product of the *LEP* gene, is secreted mainly by adipose tissue and acts as a satiety signal on the hypothalamus, thereby regulating body weight and energy expenditure (Campfield *et al.* 1995). In swine, leptin mRNA levels are greater in adipose tissue from obese pigs than lean pigs (Robert *et al.* 1998; McNeel *et al.* 2000). Furthermore, injection of recombinant porcine leptin reduces feed intake and increases growth hormone (GH) secretion in swine (Barb *et al.* 1998). These observations suggest that *LEP* may be a candidate gene for economically important production traits such as backfat thickness, feed intake and growth rate in swine. The objectives of the present study were to estimate the frequency of previously reported DNA polymorphisms (Stratil *et al.* 1997; Robert *et al.* 1998) in different porcine breeds, and to investigate their association with production traits.

The polymerase chain reaction (PCR) amplifications of three genomic fragments of the porcine *LEP* gene that contain polymorphisms were performed. Primers used in the study were based on available genomic (Bidwell *et al.* 1997; GenBank SSU66254) and mRNA sequences (Robert *et al.* 1998; GenBank AF026976). The first primer pair (foward 5′-AGAGTCCAGGATGACAC-3′; reverse 5′-ATCTGTTGGT-AGATCGC-3′) amplified a region from nucleotide 976–3562 (Bidwell *et al.* 1997). The PCR reaction was carried out in a 50-μl total volume containing 500 ng of genomic DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.6 units of Expand HF PCR System enzyme mix (Roche Molecular Biochemicals, Mannheim, Germany), 1X Expand HF buffer and 0.3 μM of each primer. The PCR profile was 2 min at 95 °C; 10 cycles of 15 s at 95 °C, 30 s at 50 °C, 2 min at 68 °C; followed by 37 cycles of 15 s at 95 °C, 30 s at 52 °C, 2 min at 68 °C with an increment of 5 s for each cycle, and a final 7 min extension at 68 °C. This PCR amplification generated a fragment of 2586 bp (fragment 1).

Primers for fragment 2 (foward 5′-AGCAGTCTGTCTCC-TCCAAAC-3′; reverse 5′-AGTAATAGATGCTGATGCGCC-3′) amplified a region from nucleotides 3398 to 5480 (2082 bp total, Bidwell *et al.* 1997), whilst fragment 3 primers (foward 5′-CCCTGCTTGCACTTGGTAGC-3′; reverse 5′-CTGCCACA-CGAGTCTTGCTC-3′) amplified a region from nucleotides 2237 to 2895 (658 bp total, Robert *et al.* 1998). The PCR reactions of both fragments 2 and 3 were performed in a 100-μl total volume containing 50 ng of genomic DNA, 0.2 mM of each dNTP, 1.0 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase, 1X *Taq* polymerase buffer and 0.4 μM of each primer. The PCR profile was 2 min at 94 °C, 38 cycles of 1 min at 94 °C, 1 min at 64 °C (fragment 2) or 66 °C (fragment 3), 1 min at 72 °C, and a final 5 min extension at 72 °C.

Amplified fragments were digested with *Xba*I (fragment 1), *Bgl*II and *Hin*fI (fragment 2), or *Hin*dIII (fragment 3). Figure 1 illustrates the fragment patterns of the *Xba*I, *Bgl*II and *Hin*dIII DNA polymorphisms. Fragments corresponding to each allele of the *Xba*I, *Bgl*II and *Hin*dIII DNA polymorphisms were sequenced on an ABI PRISM 377

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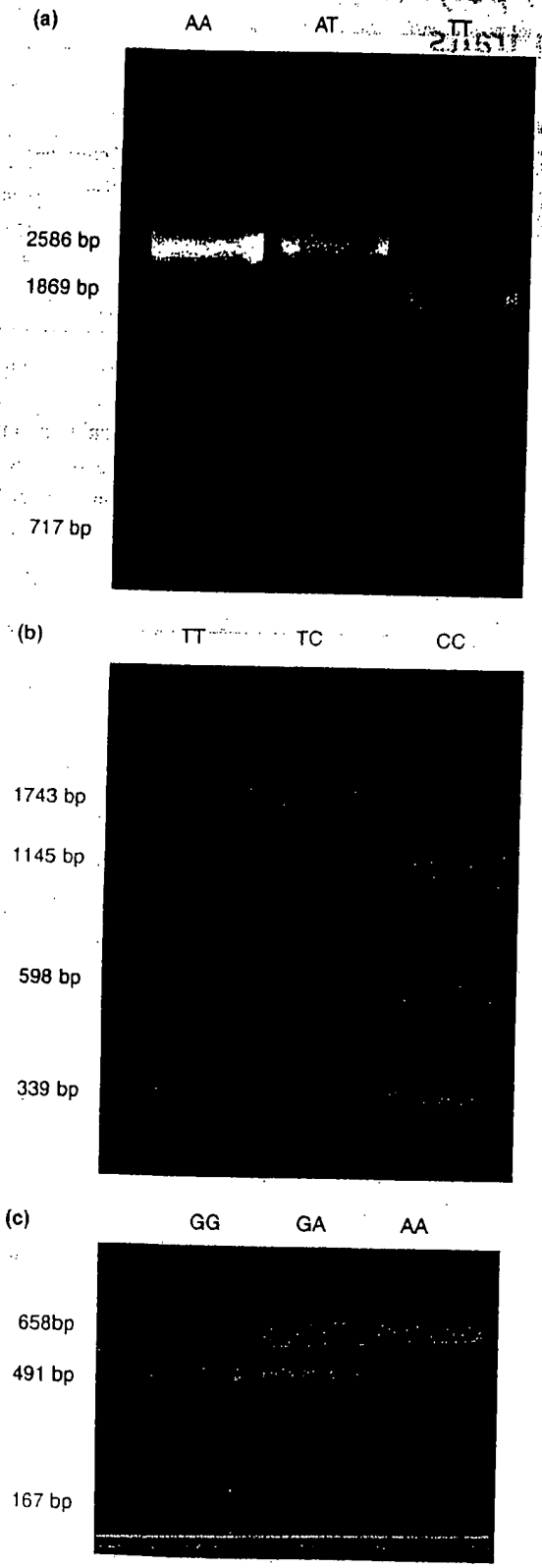

Figure 1 (a) Observed genotypes of the porcine *LEP* A2845T polymorphism after *Xba*I digestion of fragment 1: AA homozygote, 2586 bp; AT heterozygote, 2586 + 1869 + 717 bp; TT homozygote, 1869 + 717 bp. (b) Observed genotypes of the porcine *LEP* T3996C polymorphism after *Bgl*II digestion of fragment 2: TT homozygote, 1743 + 339 bp; TC heterozygote, 1743 + 1145 + 598 + 339 bp; CC homozygote, 1145 + 598 + 339 bp. (c) Observed genotypes of the porcine *LEP* G2728A polymorphism after *Hind*III digestion of fragment 3: GG homozygote, 491 + 167 bp; GA heterozygote, 658 + 491 + 167 bp; AA homozygote, 658 bp. Genotypes of individuals are indicated above the lanes.

automated DNA sequencer revealing A2845T, T3996C and G2728A polymorphisms, respectively. The A2845T substitution occurred in the second intron of the *LEP* gene whilst T3996C and G2728A substitutions were in the 3′ untranslated region (3′ UTR) region. Analyses also confirmed the *Hin*fI polymorphism identified by Stratil *et al.* (1997) and characterized by a T3469C substitution in the second exon.

Genotypes were assayed in two sample groups from the 'Programme d'Évaluation des Porcs en Station' population, kindly provided by the Centre de Développement du Porc du Québec. In the first sample, pigs were selected according to the highest and lowest estimated breeding values (EBV) for age (days; adjusted to 100 kg) and backfat thickness (mm; adjusted to 100 kg) (Kemp & Rothschild 1994), generating two subgroups (positive and negative). Backfat thickness was measured between the third and fourth last ribs, 5 cm off the mid-line of the split carcass. The positive subgroup included animals with lower genetic growth potential (more days to reach 100 kg) and higher backfat thickness values at 100 kg. The negative subgroup included animals with higher genetic growth potential and lower backfat thickness at 100 kg. Age- and fat-EBV values of 0 corresponded to the average of genetic growth potential and genetic leanness potential for all contemporary purebred pigs assessed in Canada. The positive subgroup included pigs with positive age- and backfat-EBV (age-EBV ≥ 0.9; backfat-EBV ≥ 0.2) whilst the negative subgroup included pigs with negative age- and backfat-EBV (age-EBV ≤ -1.6; backfat-EBV ≤ -0.3). In total, the first sample included 182 unrelated pigs separated by two generations, including 46 Duroc, 58 Landrace and 78 Yorkshire. This pig sample was analysed using Fisher's exact test (two-tailed).

The second pig sample of 182 pigs included randomly selected Duroc ($n = 40$) and Yorkshire ($n = 40$) pigs and all available Landrace pigs ($n = 102$). Production traits measured on these animals for the growing period (30–105 kg) included average daily weight gain (kg/day), feed conversion ratio (kg of feed/kg of weight gain), total feed intake (kg), daily feed intake (kg), backfat-EBV (mm, adjusted to

100 kg), age-EBV (days, adjusted to 100 kg), backfat adjusted to 100 kg (mm) and age adjusted to 100 kg (days). This second pig sample was analysed with SAS (SAS Institute Inc. 1989) using weighted ANOVA with the general linear models (GLM) procedure and for each trait, the model included sex and genotype as fixed effects using a stringent threshold for significance ($P < 0.01$).

In the first sample of pigs, there were significant differences in allelic frequencies between positive and negative subgroups of Landrace pigs for the A2845T, G2728A and T3469C polymorphisms (Table 1). These allelic frequencies were higher in the Landrace positive EBV subgroup compared with the Landrace negative EBV group, suggesting a negative effect of these alleles on backfat- and age-EBV. In the Yorkshire breed, the T, C and A alleles at position 2845, 3996 and 2728, respectively, were fixed, whilst the frequency of the C allele at position 3469 did not differ between negative and positive EBV subgroups. Analysis of the second sample indicates that frequencies of the T, C, A and C alleles at positions 2845, 3996, 2728 and 3469, respectively, were low in the Duroc and Landrace breeds whilst the T (2845), C (3996) and A (2728) alleles were absent in Yorkshire pigs, as was found in the first sample (Table 2).

In the second sample, weighted ANOVA revealed an association between the A2845T polymorphism and total feed intake ($P = 0.0061$) in the Landrace breed, with TT homozygotes having an average total feed intake of 218.72 ± 3.87 kg compared with 204.92 ± 1.69 kg for the AA homozygotes. Also in the Landrace breed, an association was observed between the A2845T polymorphism and age-EBV, with TT homozygotes having an average age-EBV of 4.28 ± 0.68 days compared with 1.37 ± 0.47 days for the AA homozygotes. Analyses of the *HinfI* polymorphism revealed an association between the T3469C polymorphism and average daily weight gain ($P = 0.0078$) for the Landrace breed. TT homozygote pigs had an average daily weight gain of 0.91 ± 0.011 kg/day compared with 0.85 ± 0.019 kg/day for TC heterozygotes pigs. AT heterozygous and CC homozygous estimates are not given for these polymorphisms (A2845T and T3469C, respectively) because only limited animal numbers were available for the statistical analysis.

Although these mutations are not located in the leptin coding sequence, it is possible that they affect mRNA stability or translation efficiency, resulting in specific biological effects. Polymorphisms reported may also act as molecular markers linked to a specific locus which controls growth rate and feed intake traits. The lack of association between production traits and the DNA polymorphisms in the Duroc and Yorkshire breeds suggests that linkage disequilibrium is unique to the Landrace breed. Although all available Landrace pigs ($n = 102$) of the test programme were included in the second sample, a limited number of these pigs carried the deleterious alleles. Moreover, as the polymorphisms seem to be associated with negative production traits (decreased growth rate and increased feed intake), it is not surprising to see low frequencies of these mutant alleles. Indeed, selection pressure which has been maintained to increase pig growth

Table 1 Allelic frequencies of porcine *LEP* for negative and positive subgroups of the first sample of pigs.

Polymorphism	Breed	No. of tested pigs (neg/pos)	Allelic frequency[1]		
			Negative[2]	Positive[3]	P^4
A2845T	Duroc	26/14	13.5	7.1	0.483
	Landrace	43/13	11.6	34.6	**0.014**
	Yorkshire	39/28	0	0	1
T3996C	Duroc	27/17	3.7	5.9	0.638
	Landrace	44/14	10.2	25	0.062
	Yorkshire	51/27	0	0	1
G2728A	Duroc	28/18	3.6	2.8	1
	Landrace	44/14	9.1	25	**0.048**
	Yorkshire	42/28	0	0	1
T3469C	Duroc	27/17	1.9	2.9	1
	Landrace	44/13	3.4	15.4	**0.046**
	Yorkshire	50/26	8	11.5	0.386

[1] Frequency of T allele for A2845T, C allele for T3996C, A allele for G2728A and C allele for T3469C.
[2] Age EBV ≤ −1.6; backfat EBV ≤ −0.3.
[3] Age EBV ≥ 0.9; backfat EBV ≥ 0.2.
[4] Fisher exact test (two-tailed) probability.
$P < 0.05$ are given in bold.

Table 2 Allelic frequencies of porcine *LEP* polymorphisms and associations with production traits assessed on the second sample of pigs.

Polymorphism	Breed	No. of tested pigs	Allelic frequency		Association between genotype and traits[1]							
			ATGT	tcac	ADWG	FC	TFI	DFI	Backfat EBV	Age EBV	Backfat	Age
A2845T	Duroc	39	0.68	0.32	0.29	0.51	0.74	0.32	0.65	0.08	0.77	0.16
	Landrace	102	0.93	0.07	0.35	0.83	**0.006**	0.61	0.69	**0.003**	0.83	0.52
	Yorkshire	40	1	0								
T3996C	Duroc	40	0.89	0.11	0.42	0.90	0.61	0.45	0.09	0.31	0.39	0.65
	Landrace	102	0.85	0.15	0.84	0.89	0.41	0.75	0.03	0.02	0.20	0.59
	Yorkshire	40	1	0								
G2728A	Duroc	40	0.91	0.09	0.48	0.88	0.81	0.50	0.06	0.49	0.66	0.71
	Landrace	102	0.85	0.15	0.84	0.89	0.41	0.75	0.03	0.02	0.20	0.59
	Yorkshire	40	1	0								
T3469C	Duroc	40	0.91	0.09	0.48	0.88	0.81	0.50	0.06	0.49	0.66	0.71
	Landrace	102	0.94	0.06	**0.008**	0.36	0.28	0.14	0.61	0.33	0.61	0.07
	Yorkshire	40	0.85	0.15	0.39	0.26	0.96	0.99	0.75	0.74	0.47	0.59

[1] Weighted ANOVA probabilities for average daily weight gain (ADWG), feed conversion (FC), total feed intake (TFI), daily feed intake (DFI), backfat EBV, age EBV, backfat (100 kg) and age (100 kg). $P < 0.01$ are given in bold.

rate and decrease backfat thickness over the past 20 years may contribute to the elimination or decrease of unfavourable alleles such as these.

Acknowledgements

We thank Steve Méthot for statistical analysis, Joël Rivest for database assistance and Lynn Lessard for manuscript revision. This work was financially supported by the Fédération des Producteurs de Porcs du Québec, Centre de Développement du Porc du Québec, Ontario Swine Improvement and Agriculture and Agri-Food Canada. Lennoxville contribution no. 689.

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First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/932,888	
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Title	:	Porcine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0016	

EXHIBIT B

OF

AMENDMENT AFTER FINAL

Hybridization with Analysis of DNA Blots.
Current Protocols in Molecular Biology,
Section II 2.10.1. (John Wiley & Sons 2000)

Hybridization Analysis of DNA Blots

The principle of hybridization analysis is that a single-stranded DNA or RNA molecule of defined sequence (the "probe") can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"), with the stability of the hybrid depending on the extent of base pairing that occurs. Experimentally, the analysis is usually carried out with a probe that has been labeled and target DNA that has been immobilized on a membrane support. Hybridization analysis is sensitive and permits detection of single-copy genes in complex genomes. The technique has widespread applications in molecular biology.

The first stage in a hybridization experiment is to immobilize the denatured nucleic acids on a suitable solid support. Methods for achieving this with gel-fractionated and bulk DNA are described in *UNITS 2.9A & 2.9B*. The labeled probe is then applied in a solution that promotes hybridization. After a suitable incubation, the membrane is washed so that any nonspecifically bound probe is removed, leaving only probe that is base-paired to the target DNA. By controlling the stringency of the washing conditions, decisions can be made about whether to target sequences that are 100% complementary to the probe, or allow some mismatching so that sequences with lower degrees of similarity are also detected. The latter approach (heterologous probing) is used to study related sequences in a single or more than one genome.

Hybridization analysis was originally carried out with long (100 to 1000 bp), radioactively labeled DNA probes. Other types of probe (RNA, oligonucleotide) have more recently been introduced, as have nonradioactive labeling and detection strategies. In addition, improvements in understanding of the factors that influence hybrid stability and hybridization rate have led to a proliferation of reagents and protocols for hybridization analysis. Finding one's way through the maze can be a daunting task, especially as protocols that work well with one probe-target combination may not work so well if either member of the partnership is changed. The approach taken here is to present as the basic protocol an unsophisticated procedure for hybridization analysis with a radiolabeled DNA probe. Despite its lack of embellishments, the protocol gives acceptable results with Southern and dot blots on nitrocellulose and nylon (uncharged and charged) membranes. The alternate protocol describes a similar method for probing DNA blots with a radiolabeled RNA probe. A support protocol for stripping blots to ready them for reprobing is also provided.

Relevant units elsewhere in the manual include the following: *UNITS 3.18 & 3.19* describe the preparation of nonradioactive probes and their use in hybridization analysis; *UNIT 4.9* covers hybridization analysis of immobilized RNA; *UNIT 6.3* describes hybridization analysis of recombinant clone libraries; and *UNIT 6.4* explains how to use labeled oligonucleotides as hybridization probes.

These hybridization protocols should not be read in isolation. The commentary describes various modifications that can be introduced, including changes to prehybridization, hybridization, and wash solution formulations, and alterations to incubation times and conditions, the latter including a discussion of the wash conditions compatible with different degrees of stringency. The intention is provide the reader with sufficient data to make well-informed decisions about how to modify the basic and alternate protocols for specific applications.

CAUTION: Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves and their clothing. When working with ^{32}P, investigators should frequently check themselves and the working area for

Contributed by Terry Brown

Current Protocols in Molecular Biology (1993) 2.10.1-2.10.16

radioactivity using a hand-held monitor. Any radioactive contamination should be cleaned up using appropriate procedures. Radioactive waste should be placed in appropriately designated areas for disposal. Follow the guidelines provided by your local radiation safety adviser.

HYBRIDIZATION ANALYSIS OF A DNA BLOT WITH A RADIOLABELED DNA PROBE

BASIC PROTOCOL

This protocol is suitable for hybridization analysis of Southern transfers (*UNIT 2.9A*) and dot and slot blots (*UNIT 2.9B*) with a radioactively labeled DNA probe 100 to 1000 bp in length. The steps employ nylon membranes (uncharged or positively charged) but are suitable for nitrocellulose if modified as described in the annotations. The commentary describes how to tailor the protocol for individual requirements.

A hybridization experiment can be divided into three stages. First, the membrane is incubated in a prehybridization solution containing reagents that block nonspecific DNA binding sites on its surface, thereby reducing background hybridization. In this protocol, the blocking agents are Denhardt solution and denatured salmon sperm DNA; alternatives are described in the commentary. In the second stage, the prehybridization solution is replaced by fresh buffer containing the labeled probe, and an overnight incubation is carried out to allow the probe to bind to target sequences in the immobilized DNA. During this hybridization step, the probe pairs not only with target sites that have 100% complementarity with the probe, but also with related sequences. In the final stage of the experiment the membrane is washed with a series of solutions that gradually remove bound probe molecules until only highly matched hybrids remain.

Materials

DNA to be used as probe
Aqueous prehybridization/hybridization (APH) solution, room temperature and 68°C
2× SSC/0.1% (w/v) SDS
0.2× SSC/0.1% (w/v) SDS, room temperature and 42°C
0.1× SSC/0.1% (w/v) SDS, 68°C
2× and 6× SSC (*APPENDIX 2*)

Hybridization oven (e.g., Hybridiser HB-1, Techne) *or* 68°C water bath or incubator
Hybridization tube *or* sealable bag and heat sealer

Additional reagents and equipment for DNA labeling by nick translation or random oligonucleotide priming (*UNIT 3.5*), measuring the specific activity of labeled DNA and separating unincorporated nucleotides from labeled DNA (*UNIT 3.4*), and autoradiography (*APPENDIX 3*)

1. Label the probe DNA to a specific activity of $>1 \times 10^8$ dpm/μg by nick translation or random oligonucleotide priming. Measure the specific activity and remove unincorporated nucleotides.

   The probe should be a double-stranded DNA fragment, ideally 100 to 1000 bp in length. Usually the probe DNA is obtained as a cloned fragment (Chapter 1) which is purified from the vector by restriction digestion (UNIT 3.1) followed by recovery from an agarose gel (UNIT 2.6).

2. Wet the membrane carrying the immobilized DNA in 6× SSC.

   The membrane is blotted as described in UNIT 2.9A. Do not handle the membrane: use clean blunt-ended forceps.

3. Place the membrane, DNA-side-up, in a hybridization tube and add ~1 ml APH solution per 10 cm^2 of membrane.

   Prehybridization and hybridization are usually carried out in glass tubes in a commercial hybridization oven. Alternatively, a heat-sealable polyethylene bag can be used. The membrane should be placed in the bag, all edges sealed using a heat sealer, and a corner cut off. The APH solution is then pipetted into the bag through the cut corner and resealed.

4. Place the tube in the hybridization oven and incubate 3 hr with rotation at 68°C.

   If using a bag, shake slowly in a suitable incubator or water bath.

   If using a nylon membrane, reduce the prehybridization period to 15 min, but warm the prehybridization/hybridization solution to 68°C before adding to the membrane.

5. Denature the probe DNA by heating for 10 min in a water bath or incubator at 100°C. Place in ice.

   Step 5 should be done immediately before step 6, with a minimum delay between them.

6. Pour the APH solution from the hybridization tube and replace with an equal volume of prewarmed (68°C) APH solution. Add denatured probe and incubate with rotation overnight at 68°C.

   The probe concentration in the hybridization solution should be 10 ng/ml if the specific activity is 10^8 dpm/μg, or 2 ng/ml if the specific activity is 1×10^9 dpm/μg. If using a bag, cut off a corner, pour out the prehybridization solution, add the hybridization solution plus probe, and reseal. It is very difficult to avoid contaminating the bag sealer with radioactivity; furthermore, the sealing element (the part that gets contaminated) is often difficult to clean. Hybridization bags are therefore not recommended.

7. Pour out the APH solution, using the appropriate disposal method for radioactive waste, and add an equal volume of 2× SSC/0.1% SDS. Incubate with rotation for 10 min at room temperature, changing the wash solution after 5 min.

   CAUTION: *All wash solutions must be treated as radioactive waste and disposed of appropriately.*

   To reduce background, it may be beneficial to increase the volume of the wash solutions by 100%. If using a bag, transfer the membrane to a plastic box for the washes.

8. Replace the wash solution with an equal volume of 0.2× SSC/0.1% SDS and incubate with rotation 10 min at room temperature, changing the wash solution after 5 min (this is a low-stringency wash; see commentary).

9. If desired, carry out two further washes as described in step 8 using prewarmed (42°C) 0.2× SSC/0.1% SDS for 15 min each at 42°C (moderate-stringency wash).

10. If desired, carry out two further washes using prewarmed (68°C) 0.1× SSC/0.1% SDS for 15 min each at 68°C (high-stringency wash).

11. Pour off the final wash solution, rinse the membrane in 2× SSC at room temperature, and blot excess liquid. Wrap in plastic wrap.

    Do not allow the membrane to dry out if it is to be reprobed.

12. Set up an autoradiograph (*APPENDIX 3*).

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/932,888	
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EXHIBIT C

OF

AMENDMENT AFTER FINAL

Nucleic Acid Hybridization, pp 8-1 to 8-4, obtained at http://dir.niehs.nih.gov/dirlep/files/hybridiz.pdf on 11-19-04

NUCLEIC ACID HYBRIDIZATION

DNA can be denatured and renatured. The process of strand separation is called denaturation (colloquially called melting). Heating or exposure to low salt concentrations destabilizes the noncovalent forces that stabilize the double helix, and this results in strand spearation. DNA denaturation occurs over a narrow temperature range. For a particular DNA molecule, the melting temperature is influenced by the proportion of GC base pairs. The more GC base pairs, the higher the temperature necessary to denature the DNA.

If conditions are brought back to normal (e.g., physiological temperature), the single stranded (denatured) DNA can renature or reform complementary strands according to the rules of base pairing (A pairs with T and G pairs with C). When the renatured DNA strands are precisely complementary, the original double stranded helical structure can reform. These same biological features of DNA make it possible to manipulate nucleic acids in vitro.

When any two nucleic acids pair together by virtue of complementarity they are said to anneal with each other and form a duplex structure. When the nucleic acids are from different sources, as occurs when one preparation consists of DNA and the other RNA, the annealing process is described as hybridization. The two common ways of performing these reactions in vitro are solution hybridization and filter (or solid support) hybridization.

Nucleic acid hybridization - the formation of a duplex between two complementary sequences, usually between two molecules that have complementary bases. It is possible for a single strand of nucleic acid that has inverted repeat sequences to hybridize back onto itself forming a stem and loop structure.

DNA - DNA hybridization
DNA - RNA hybridization

Hybrid Stability

Intrinsic factors

A duplex with relatively more GC base pairs than AT base pairs
will be more stable because there are three hydrogen bonds
between G and C and only two between A and T
Thus, it would take a higher temperature to denature

A GC base pair-rich duplex

The degree of complementarity between two strands also influences stability.

Extrinsic factors (experimental conditions)

1. temperature
2. salt concentration
3. presence of denaturing agents (e.g., formamide)
4. presence of high molecular weight polymers (e.g., dextran sulfate)

Temperature

- Ideal = 25 C below duplex melting temperature
- High temperatures may damage nucleic acids

Salt concentration

- Hybridization rate increases between 0.1 M and 1.2 M
- Commonly use 5 to 6 x SCC for solid support hybridization
  - 1 x SCC = 0.15M NaCl & 0.015M sodium citrate at pH 7.2 to 7.4
- During washing the amount of SCC is lowered depending upon required stringency

Denaturing agents

- Every 1% formamide allows lowering temperature 0.7 C without losing specificity
- Concentrations of 50% or greater formamide favor DNA-RNA hybridization over DNA-DNA hybridization

High molecular weight polymers

- Effectively increase concentration of nucleic acids by excluding volume from the hybridization mixture

Stringency

- By manipulating temperature and salt concentration, one can distinguish between perfect duplexes and duplexes that have mismatches between bases
- Under stringent conditions only perfect or near perfect duplexes can be formed
- The melting temperature of a duplex decreases 1 C for every mismatched base pair
- Relaxed conditions that allow duplex formation with mismatched base pairs include lowering the temperature

Stability of duplexes with mismatched base pairs is favored by a higher salt concentration
Wash conditions on solid supports can be adjusted to achieve the desired amount of stringency
Under stringent conditions, wash temperature can be increased and salt concentration can be decreased (down to 0.1 x SCC)

Solid Support Hybridizations

Denatured DNA or RNA is immobilized on an inert support (filter hybridization)
Prevents self-annealing
Bound sequences available for hybridization with an added nucleic acid (the **probe**)
Support filters
Nitrocellulose filters (most commonly used)
Nylon membrances (less brittle than nitrocellulose)
Cellulose paper impregnated with diazo groups
Diazo groups covalently bind to guanine residues on the DNA or RNA to stabilize support

Types of solid support hybridizations
Dot/Slot blots
Southern (DNA) **blots**
Northern (RNA) **blots**

Dot/Slot blots
DNA or RNA is bound directly to the solid support filter and then hybridized to the probe
Good for multiple samples and quantitative measurements
Specificity for qualitative measurements may be a problem for close but not identical sequences

Specific Hybridization Applications

In situ Hybridization
FISH
Molecular Arrays

Hybridization Probes and Methologies

Radioactive versus nonradioactive
Purified insert versus vector
Labeling methods
Nick translation

T4 DNA polymerase
End-labeling with T4 polynucleotide kinase
End-labeling with terminal deoxynucleotidyl transferase
End-labeling with the Klenow fragment of E. coli DNA polymerase
Random primer
Polymerase chain reaction
Riboprobes
Removal of unincorporated label after probe preparation
Use of oligonucleotides
Denatured double-stranded DNA probes